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TECHNICAL MANUSCRIPT 111

THE EFFECT OF MOISTURE ON ETHYLENE OXIDE STERILIZATION

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UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK

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THE EFFECT OF MOISTURE ON ETHYLENE OXIDE STERILIZATION

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ABSTRACT

Bacterial cells once dehydrated beyond a critical point no longer react uniformly to ethylene oxide sterilization procedures. The percentage of cells that are resistant to the lethal effect of ethylene oxide after cell desiccation is often small, sometimes only 0.001 to 0.01 per cent. However, five per cent resistant cells have been observed with one type microorganism dried in broth. The presence of organic matter increases the percentage of cells that become resistant to ethylene oxide after dehydration. The phenomenon is produced by exposing cells to a vacuum or a chemically desiccated atmosphere. It is not a permanent change, as the resistant cells rapidly become normal if directly wetted with water. On the other hand, mere exposure to a high relative humidity (75 to 98 per cent) following desiccation requires six and four days, respectively, to overcome this resistant effect. Moisture content studies show there to be less water in bacterial cells that have been desiccated and then equilibrated to successively higher relative humidities up to 100 per cent, than in cells that have not been desiccated, but allowed to dry naturally until equilibrated to the same relative humidities.

I. INTRODUCTION

Dry sterilization with ethylene oxide gas has achieved importance only within the past decade. It was possible in 1949 to publish a review on this subject,[1] but at that time the technique had few applications. The fact that ethylene oxide is bactericidal in the vapor state was then relatively unknown, even though several patents and a number of papers had been published on the subject.[2-6] Almost all applications of ethylene oxide as a sterilizing agent had been in treating dry foodstuffs, particularly spices. With the review, there was published a summary of six years' research on ethylene oxide[7-9] that indicated that the effects of time, temperature, and concentration upon the sterilization process were uncomplicated. This has been substantiated by the considerable practical experience with the process accumulated in the last decade.

Phillips, in 1949,[7] indicated that the effect of extraneous organic material and variation between bacterial species[7-10] was of less importance with ethylene oxide than with other chemical disinfectants. This, too, has been borne out by experience.

This "dry" sterilization process, however, is dry only in the sense that liquid water is not involved. The ethylene oxide sterilization process is not carried out under completely anhydrous conditions, particularly in nonevacuated systems. In practice, some water vapor is present in the air surrounding the material being sterilized. Thus, the microorganisms have a moisture content that varies with the relative humidity of the atmosphere and the conditions prevailing at the time of sterilization.

Early investigators had divergent views on the effect of this moisture in ethylene oxide sterilization[1] and the first publication on this subject from these laboratories[9] pointed out that the phenomenon was complicated. Experience over the past decade has confirmed this finding.[11-17] Our study was undertaken when it became evident that most of the reported difficulties with ethylene oxide sterilization could be traced to a lack of understanding of the role of moisture, particularly when highly desiccated or lyophylized material, such as blood plasma, was involved. It became apparent that not only was the relative humidity of the atmosphere during the ethylene oxide treatment important, but also that the moisture conditions to which the microorganisms had been subjected prior to exposure were, if anything, even more important in their effects.

The following factors were investigated in this study:

1. The effect of ethylene oxide at varying relative humidities on microorganisms preconditioned to the same or different relative humidities.

2. The effect of dehydration of bacterial spores by chemical desiccation or vacuum on the rate at which they are killed by ethylene oxide at various relative humidities.

3. The time and relative humidity of ethylene oxide exposure required to overcome the resistance developed in desiccated microorganisms.

4. The effect that the nature of the supporting surface has on the rate at which microorganisms are killed by ethylene oxide at various relative humidities.

5. The effect of desiccation of various microbial species on the rate at which they are inactivated by ethylene oxide.

6. The effect of organic matter surrounding the microorganisms during desiccation on the rate at which they are killed by ethylene oxide at various relative humidities.

7. The effect of ethylene oxide concentration on the rate at which desiccated microorganisms are killed.

8. Means of breaking the resistance to ethylene oxide in desiccated cells.

II. EXPERIMENTAL METHODS

A. PREPARATION OF TEST MICROORGANISMS AND SAMPLES

The activity of ethylene oxide was demonstrated by the rate at which microorganisms were killed on surfaces when exposed to the chemical at various regulated relative humidities.

The major portion of this investigation was performed with Bacillus subtilis var. niger spores; however, a few supporting experiments were performed with Staphylococcus aureus, Mycobacterium smegmatis, Aspergillus fumigatus and T-1 bacteriophage. The B. subtilis spores were washed, resuspended in water, and then heat shocked at 60°C for 30 minutes to kill any vegetative cells. A drop of this spore suspension was placed on each of a number of clean 5/8-inch-diameter cotton cloth patches or Whatman No. 42 filter paper, and stored at a predetermined relative humidity and room temperature. The concentration of viable spores in the suspension

was regulated so that 500,000 to 5,000,000 organisms could be recovered from a patch. Aside from the cloth, Whatman ashless filter paper, glass, and Type 5 Chemical Corps filter paper were also used as test surfaces to hold the microorganisms during treatment.

The S. aureus and M. smegmatis were grown in tryptose broth for 24 hours and a drop of this broth suspension was placed on each of a number of patches that were in turn treated as described for the B. subtilis spores. The A. fumigatus spores were grown in tryptose agar, suspended in water containing Tween 20 (a nonionic wetting agent), and treated like the other microorganisms. The T-1 bacteriophage was obtained by growing E. coli in nutrient broth, inoculating with phage, and shaking for four hours. The phage was then harvested and purified by differential centrifugation. Patches were then inoculated with the purified virus for test purposes.

B. CONSTANT-HUMIDITY JARS

Before exposure to ethylene oxide, the contaminated patches were conditioned in standard desiccators to constant humidities maintained by saturated salt solutions at 25°C. A series of such desiccators was set up with various saturated salt solutions to maintain the relative humidities (RH) noted below:

Saturated Salt Solution	Relative Humidity (Per Cent)
Potassium dichromate	98
Potassium chloride	85
Sodium chloride	75
Nickel chloride	53
Magnesium chloride	33
Potassium acetate	22
Lithium chloride	11

Anhydrous calcium sulphate was used in another desiccator to achieve a relative humidity of less than one per cent.

C. ETHYLENE OXIDE EXPOSURE CHAMBER

All exposures to ethylene oxide were carried out in desiccators at 25°C. These desiccators were the vacuum type, but with the top modified (Figure 1) so that an air stream could be flushed through the jar. Contaminated patches that were to be exposed to ethylene oxide were quickly transferred from the preconditioning constant-humidity jars to these chambers and then a stream of air at the desired relative humidity was flushed through the system. The relative humidity of this air was controlled by mixing two air streams in the proper proportions. One air stream had essentially zero RH by virtue of having passed through a tube of dry calcium sulphate crystals; the other air stream had been bubbled through a column of water so that it had essentially 100 per cent RH. After the blended air was flushed through the desiccator, it passed over wet and dry bulb thermometers so that the relative humidity could be determined and adjusted. Once the desired relative humidity was achieved, the air stream was shut off and enough of the air was removed from the chamber (pressure reduced from approximately 750 millimeters of mercury to about 700 millimeters of mercury) to allow the desired amount of ethylene oxide to vaporize into the desiccator (approximately 120 milligrams per liter). By means of chemical analysis, the concentration was checked occasionally after a run. In a few experiments, concentrations of ethylene oxide higher than 120 milligrams per liter were used.

D. METHOD OF ASSAY

Following exposure to ethylene oxide, the patches were transferred to sterile stoppered test tubes containing 10 milliliters of water with 0.01 per cent Tween 20, which aids in removing microorganisms from the patches. The tubes were shaken vigorously for about five minutes and then five milliliters, one milliliter, and a sufficient number of serial dilutions were transferred to Petri dishes. The pour plates were prepared with nutrient agar enriched with yeast extract when using B. subtilis spores, and tryptose agar when using A. fumigatus, S. aureus, and M. smegmatis. Plate counts were made after 48 hours incubation.

T-1 bacteriophage was evaluated by placing the contaminated patch in a Tween 20 blank, shaking, adding samples to tubes of melted agar containing E. coli, mixing, and overlaying on agar in a Petri dish. The plates were then incubated for five hours before counting.

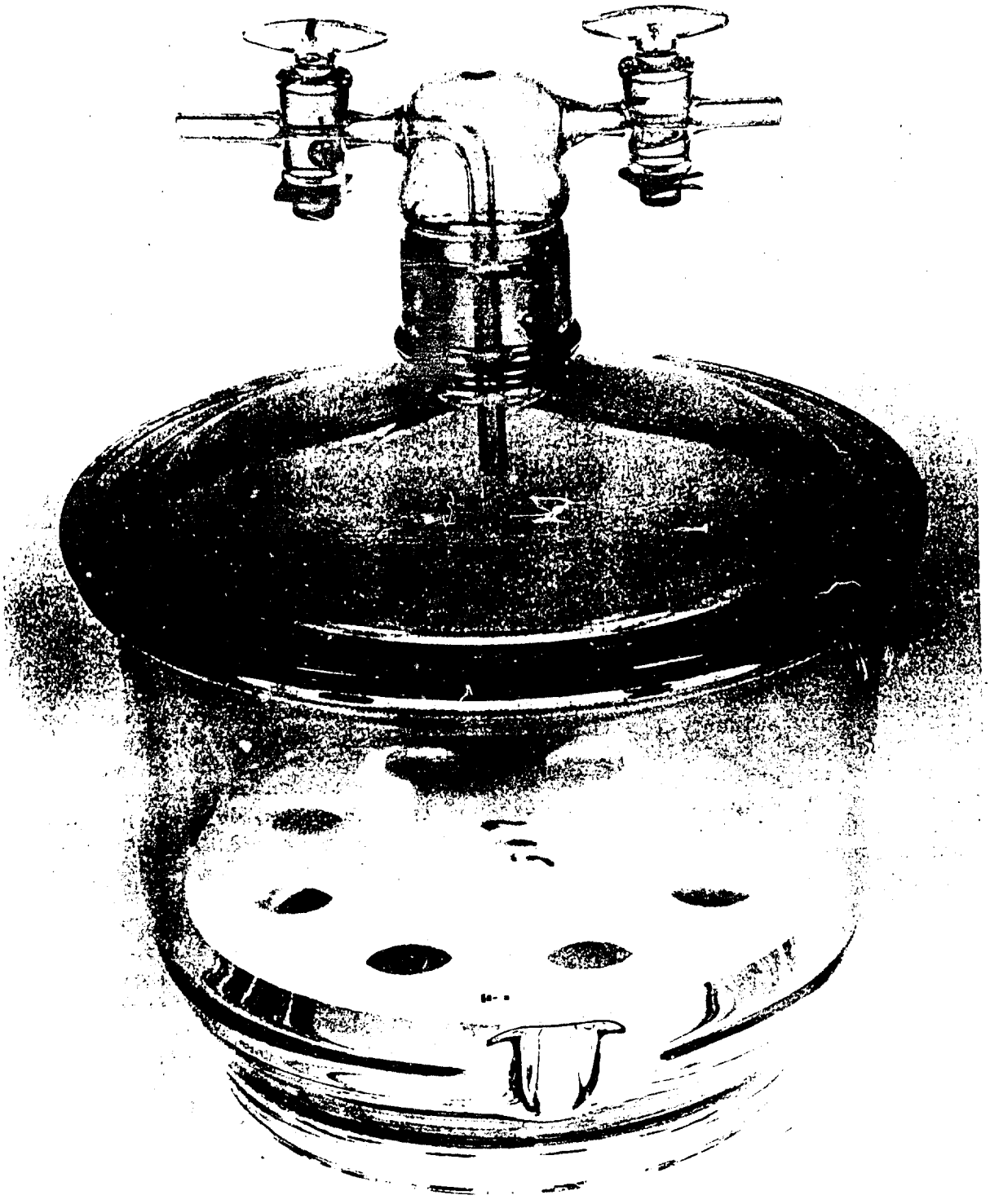

Figure 1. Ethylene Oxide Exposure Chamber. (FD Neg C-6012)

This technique, using a one-half dilution of the sample, may miss one or two residual microorganisms, but if as many as three or more organisms remain viable, not all are likely to be missed. This was particularly so in our assays, as three patches were used in each exposure, and all the runs were repeated two to four times. Each value on the graphs is thus an average of six to twelve individual observations. For the longer exposures to ethylene oxide, additional patches were used in the tests. These patches were placed directly into broth blanks and incubated to check for sterility. This all-or-nothing technique is used only as supporting evidence in our work because it gives no indication of the number of surviving organisms.

III. RESULTS

Since 33 per cent RH approaches the most effective humidity level at which to kill microorganisms on cloth patches with ethylene oxide,[1/] this same humidity was used in these studies for the reference run. The low ethylene oxide concentration (120 milligrams per liter at 25°C) was deliberately selected for these studies so that about eight hours would be required for sterilization and valid intermediate points could be obtained.

Figures 2 and 3 show the effect of relative humidity on the rate B. subtilis spores are killed by ethylene oxide. For each humidity level, the contaminated patches were dried, preconditioned, and exposed to ethylene oxide at the same relative humidity. The effect of relative humidity is very evident from these results. At the humidity values below 33 per cent, sterilization was not accomplished. A check on the retention of the ethylene oxide in the chamber for the 24- to 72-hour exposure period revealed that there was no significant loss due to leakage. Therefore, the lack of sterility at these low relative humidities could not be attributed to a lowered ethylene oxide concentration.

Figure 4 shows that preliminary drying of organisms before they are exposed to ethylene oxide at 33 per cent RH greatly increases the resistance of a small percentage of the cells to the lethal effect of the chemical. Even with a drying time as short as one hour, highly resistant microorganisms were developed, although the number was less than one in a million. The same effect was produced by exposure to vacuum (Figure 5). It may also be seen that the percentage of organisms resistant to ethylene oxide can be increased significantly by exposing the organisms to a vacuum at 54°C before treating them with ethylene oxide.

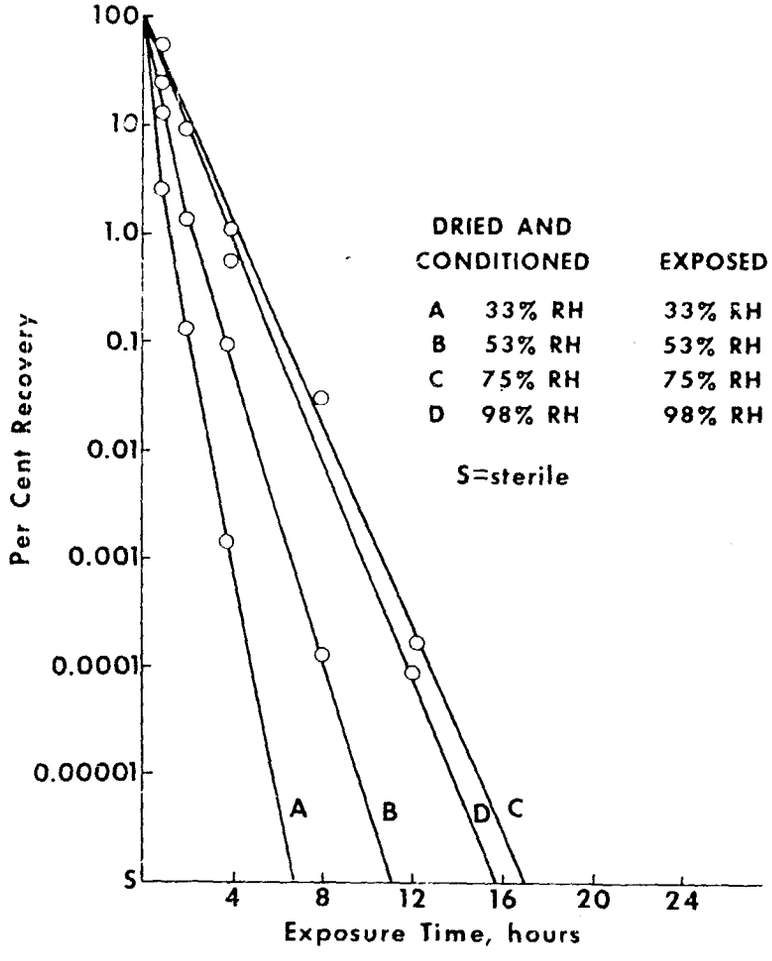

Figure 2. B. subtilis Spores on Cotton Patches Exposed to Ethylene Oxide 120 Milligrams per Liter at 25°C.

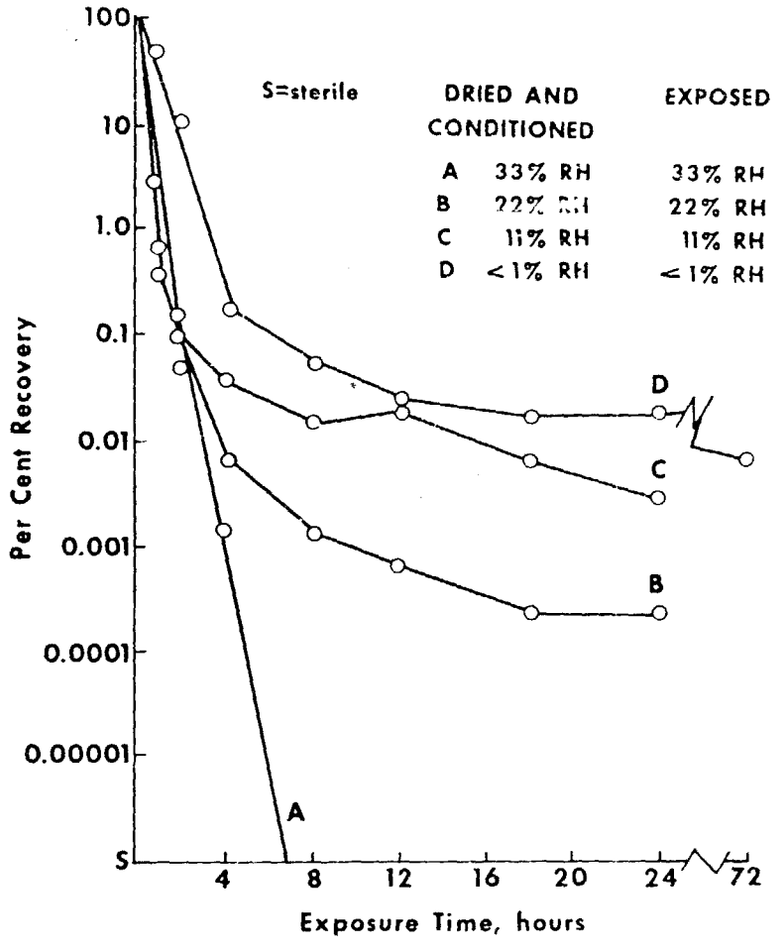

Figure 3. B. subtilis Spores on Cotton Patches Exposed to Ethylene Oxide 120 Milligrams per Liter at 25°C.

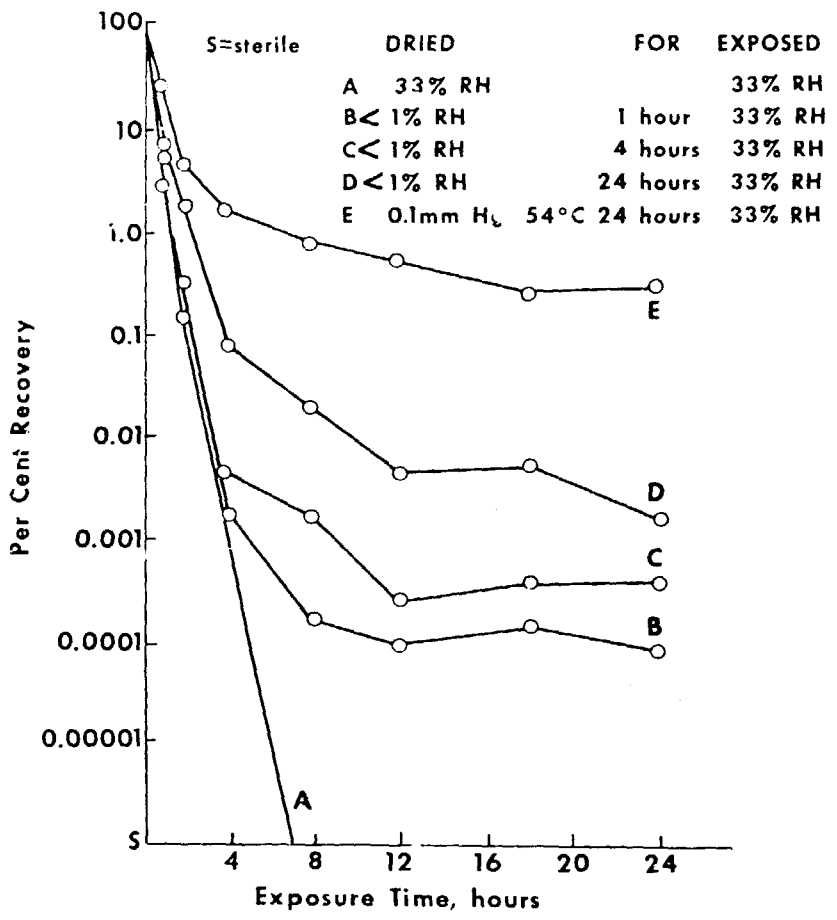

Figure 4. B. subtilis Spores on Cotton Patches Exposed to Ethylene Oxide 120 Milligrams per Liter at 25°C.

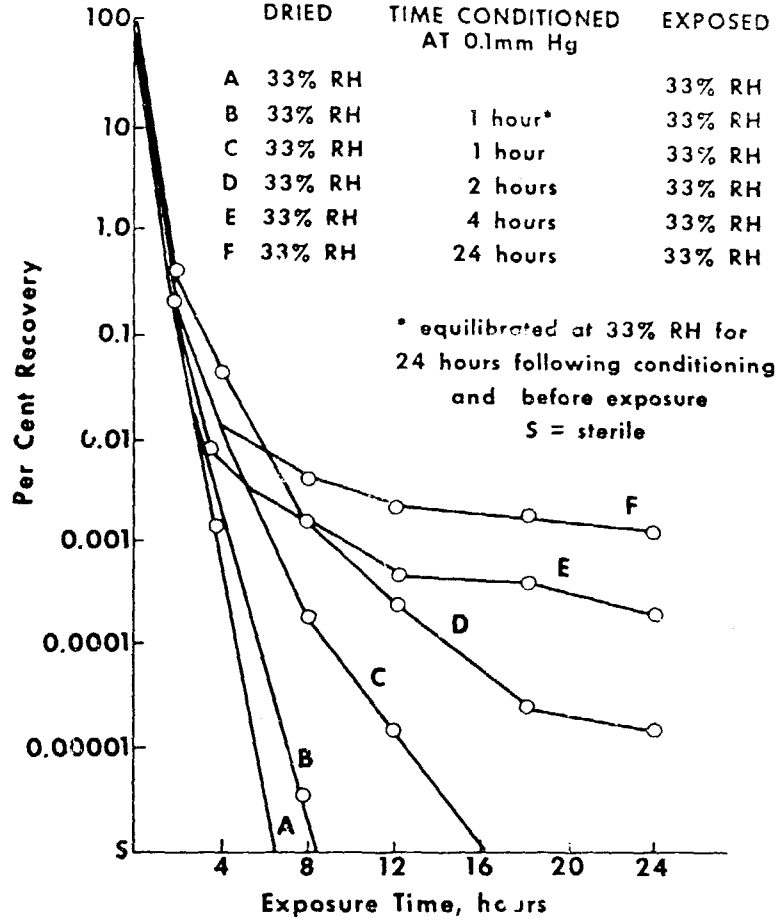

Figure 5. B. subtilis Spores on Cotton Patches Exposed to Ethylene Oxide 120 Milligrams per Liter at 25°C.

In an effort to overcome the resistance to ethylene oxide sterilization produced by dehydration, a study was made to determine the time required for these organisms to be rehydrated in various relative humidities. The results shown in Figure 6 reveal that drastic steps such as actually wetting the cells must be taken if the effect is to be overcome immediately. Merely equilibrating to a high RH (75-98 per cent) required four to six days to break the effect; it could not be overcome in twelve months' conditioning at 33 per cent RH.

Aside from the effect of desiccation, a study was also made to determine more accurately the effect of the type of surface containing the microorganisms on the rate the cells were inactivated by ethylene oxide. The results (Figure 7) reveal that it is more difficult to kill on imperious surfaces. However, the effect can be overcome, at least partially, by increasing the relative humidity at which the organisms are dried and exposed to the oxide. The Type 5 filter paper contains asbestos fibers that are imperious.

The death rates of A. fumigatus spores and cells of S. aureus, M. smegmatis and T-1 bacteriophage upon exposure to ethylene oxide after equilibration to 33 per cent and one per cent RH are shown in Figure 8. It is evident that the development of an increase in resistance to ethylene oxide sterilization on extreme drying is not peculiar to B. subtilis spores. It occurs in other bacteria, one fungus spore, and one bacteriophage as well.

The longer time required to kill vegetative cells dried from a broth medium, as compared with B. subtilis spores dried from a distilled water suspension, prompted an investigation of the effect organic matter has on the rate of death due to ethylene oxide. It is apparent from the results (Figure 9) that the vegetative cell is not more resistant than the spores if it is free of organic contamination when treated with ethylene oxide. As expected, the higher the concentration of organic matter, the slower the death rate produced by ethylene oxide. The differences in resistance due to increasing amounts of organic matter are not great, however. It was noted by Schley et al[18] that ethylene oxide is less likely to be inactivated by a large excess of organic material than the other decontaminants. Furthermore, it was shown by Phillips[10] that while there are differences in sensitivity of spores and vegetative cells to ethylene oxide, these differences are by no means as marked as with many other disinfectants.

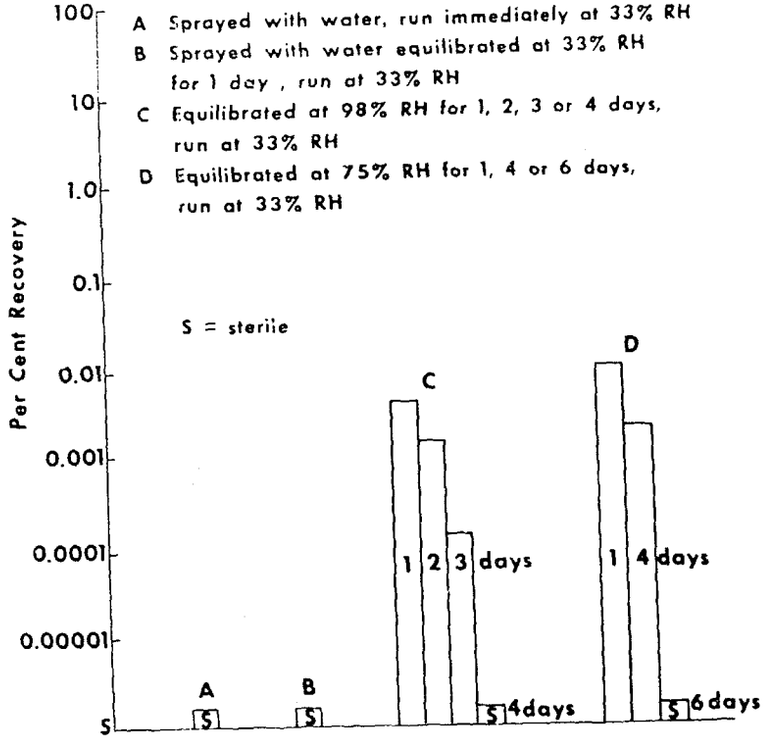

Figure 6. Rehydration Effect on B. subtilis Spores on Cotton Patches Exposed to Ethylene Oxide at 120 Milligrams per Liter at 25°C for 8 Hours. All Patches Were First Dried at Less Than 1% RH.

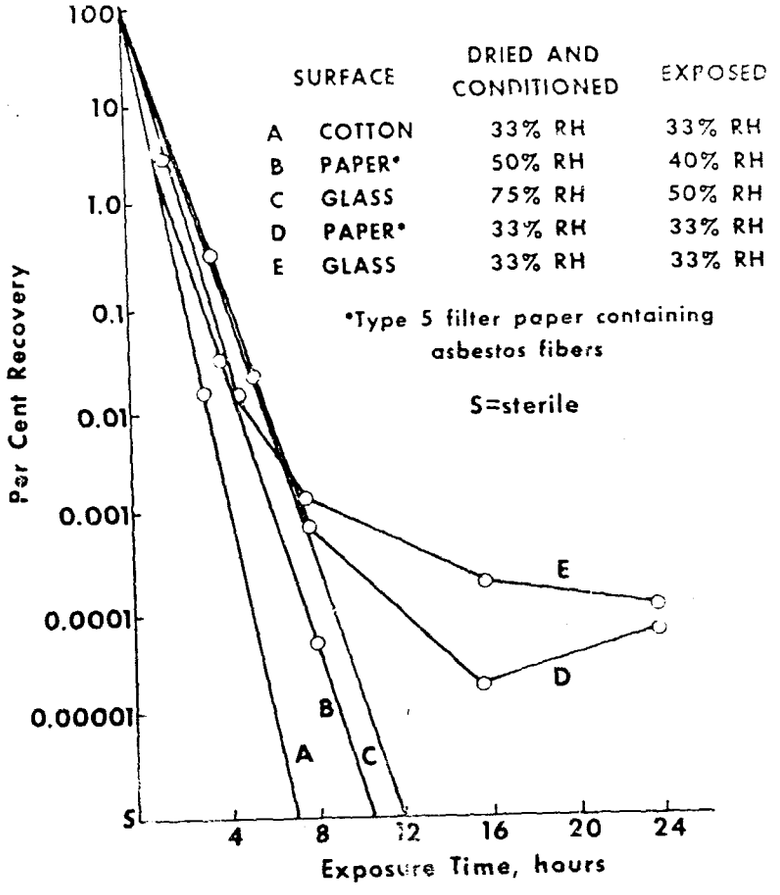

Figure 7. B. subtilis Spores on Various Surfaces Exposed to Ethylene Oxide 120 Milligrams per Liter at 25°C.

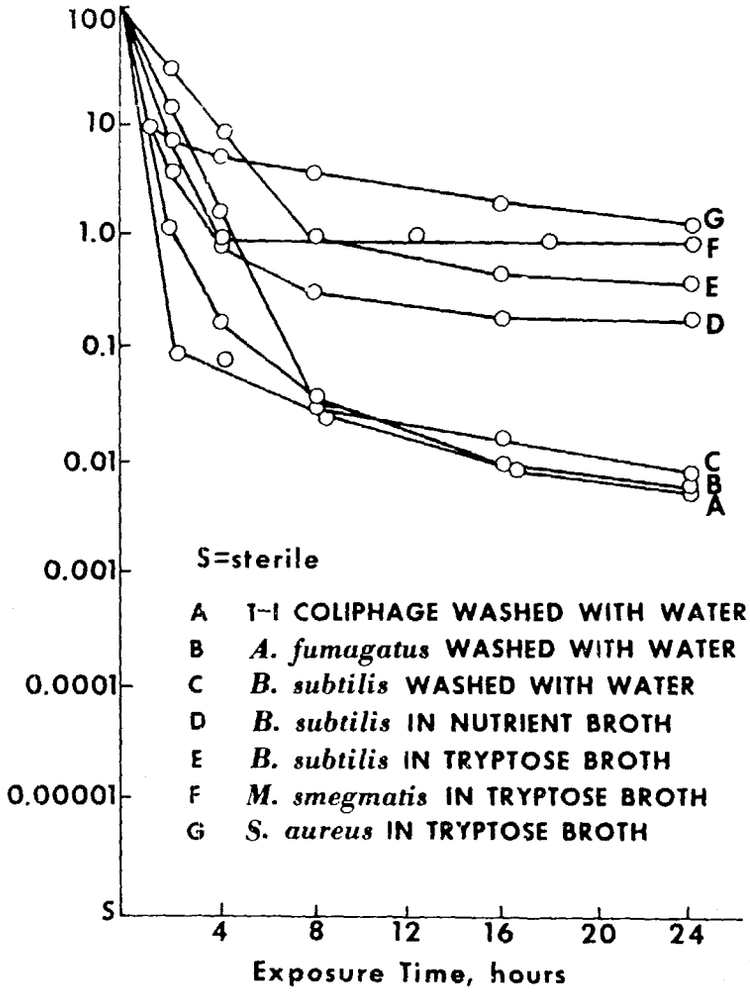

Figure 8. Various Organisms on Whatman No. 42 Filter Paper Exposed to Ethylene Oxide 120 Milligrams per Liter at 25°C. All Were Dried and Conditioned at Less Than 1% RH and Exposed at 33% RH.

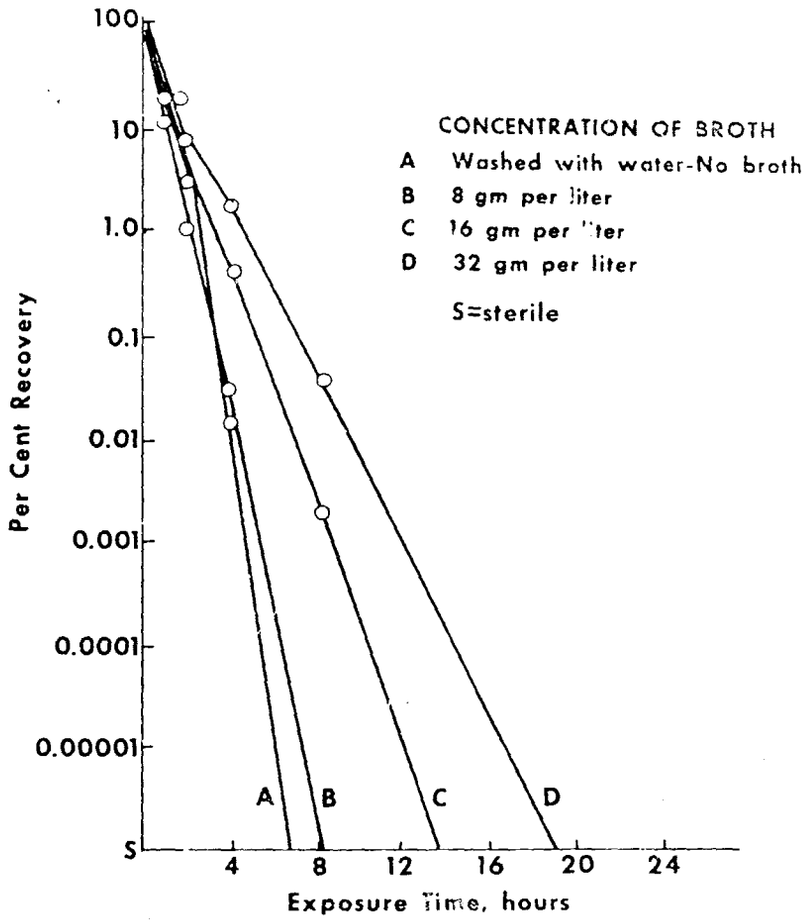

Figure 9. Staphylococcus aureus in Concentrations of Tryptose Broth Indicated, on Whatman No. 42 Filter Paper Dried and Exposed at 33% RH to Ethylene Oxide 120 Milligrams per Liter at 25°C.

All the above data were obtained in experiments employing 120 milligrams of ethylene oxide per liter of air. The effect of higher concentrations of the oxide on the death rate of previously dried B. subtilis spores is shown in Figure 10. An increase in concentration from 120 to 950 milligrams per liter increases the death rate slightly but it is not sufficient to overcome completely the resistance to ethylene oxide built up in a population of cells previously subjected to drying.

The differences in resistance to ethylene oxide of nondesiccated and once highly desiccated spores even when exposed to the oxide at the same relative humidity led to the determination of actual moisture content in the cells under the two types of condition. Figure 11 shows the per cent water in B. subtilis var. niger spores as a function of the relative humidity at 25°C. Two curves are shown; the top one is the desorption or dehydration curve and the bottom is the sorption or hydration curve. It is evident that the equilibrium moisture content of the cells is slightly but significantly different depending on whether the equilibrium was reached by hydrating thoroughly dry organisms or dehydrating wet organisms. The same difference in moisture content between hydrated and dehydrated cells had been noted earlier for Serratia marcescens.[19]

It is only when dealing with cells that have been exposed to a desiccating agent and then treated that the resistance to ethylene oxide sterilization is noted. Thus the concern is with the bottom or sorption curve in Figure 11.

IV. DISCUSSION

A large body of literature has appeared in recent years concerning the biological activity of alkylating agents.[20] One particular interest has been the mutagenic activity of these compounds and their effect on the DNA molecule. These investigations are furnishing a new insight into genetic mechanisms on a molecular scale, but the information obtained is not particularly applicable to the present problem for two reasons. First, these studies have dealt primarily with DNA and other materials in an aqueous system, but the investigation reported here is concerned with a dry system in which the chemical reaction is taking place between a gas and a microorganism. Here the whole problem is concerned with the effect of residual water in the vapor or the solid. Secondly, while mutagenic effects can only arise through action on the DNA molecule, it is by no means certain that the bactericidal action is due to alkylation on this site within the cell. It was proposed by Phillips[7,10] that the mechanism of bactericidal action of ethylene oxide was by the alkylation of various essential proteins, such as enzymes, within the cell.

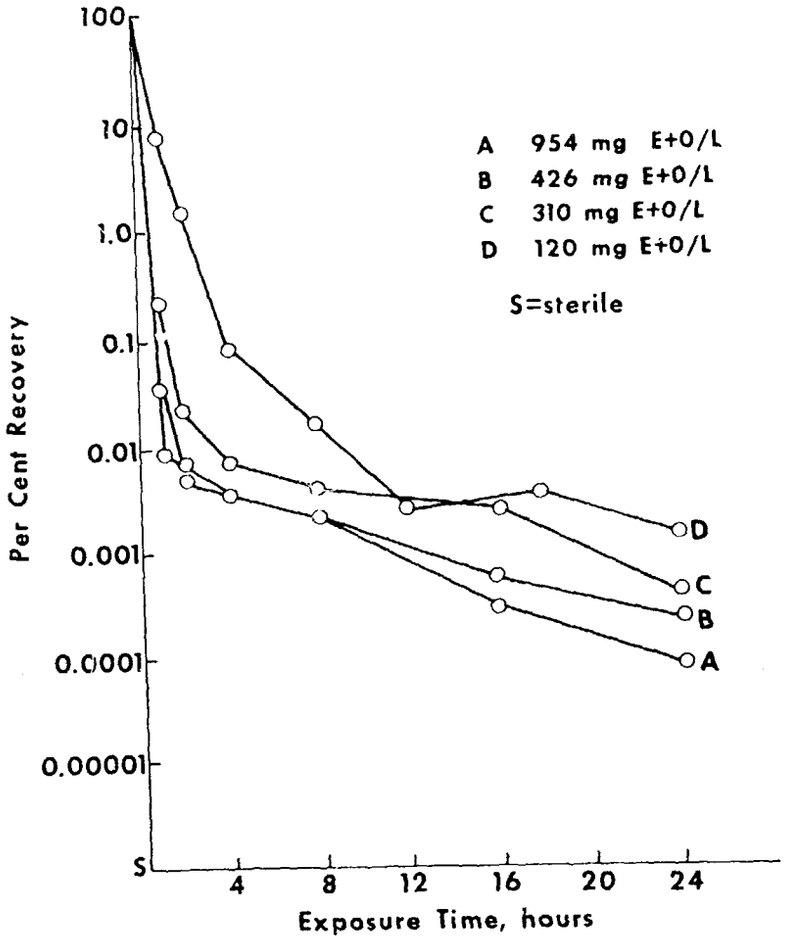

Figure 10. B. subtilis Spores on Whatman No. 42 Filter Paper Exposed to Ethylene Oxide at 33% RH and 25°C. Spores Were Previously Dried at Less Than 1% RH.

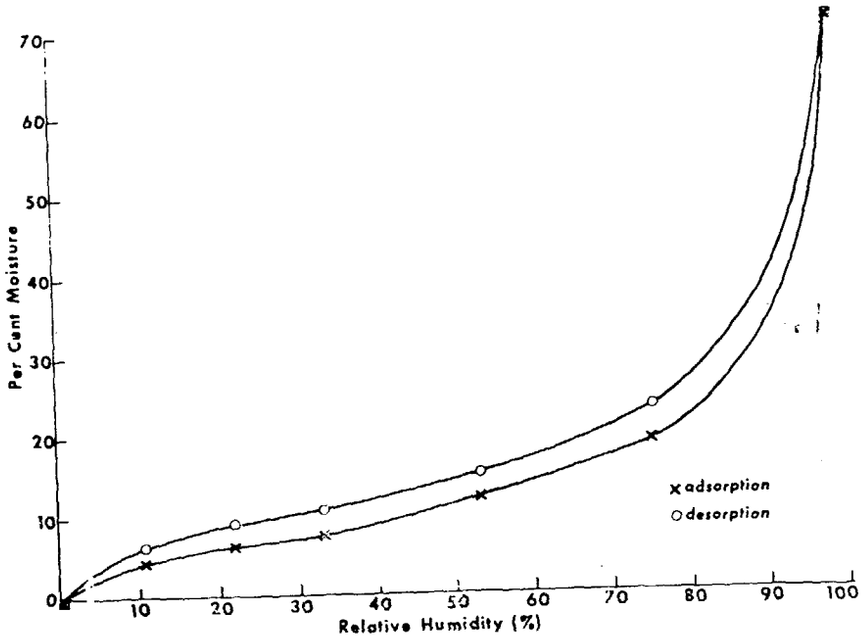

Figure 11. Moisture Content of B. subtilis Spores as a Function of Relative Humidity at 25°C.

Protein alkylation can as easily or more promptly cause death of the cell as can the induction of a lethal mutation. Indeed, both the speed of action and the observation that mutations have not been noted among surviving cells following ethylene oxide treatment would indicate that the damage to the cell by this compound is direct. If death were the result of a lethal mutation, many nonlethal mutations should have been observed. In contrast, when bacterial spores were suspended in liquid sulfur mustard, an alkylating agent whose mutagenic effect has been frequently noted of late, they survived for as long as three weeks, but no normal colonies were observed growing from treated cells toward the end of the exposure period.* We believe that we are dealing with an alkylation phenomenon, probably occurring at various rates on all positions within protein and other molecules that are capable of being alkylated, and occurring in the absence of any liquid, although all traces of water have not necessarily been removed from the system. When a sufficient number of sites within the microorganism have been alkylated, death ensues; it is not possible at this time to say exactly which sites are involved.

The particular resistance to sterilization under investigation here, which we interpret as a resistance to alkylation, occurs only when the moisture level has at some time been brought below a certain critical level, around 30 per cent RH in most of these tests; there is some indication that this critical level may vary somewhat as test conditions vary. This phenomenon of resistance induced by desiccation has been observed in all organisms investigated, but has been most closely studied with B. subtilis var. niger spores.

The resistance induced by desiccation is not a permanent one. The resistant spores react normally once fully rehydrated. This recovery is almost instantaneous if the spores are directly wetted with water, but is quite slow if the spores are exposed to a moist or even essentially saturated atmosphere. As the same cells can be switched from resistant to normal states by manipulating their internal moisture, evidently no mutation is involved, and, indeed, the resistance does not appear in the progeny of cells that have resisted sterilization.

The most interesting observation, however, is that all cells are not equally affected. Where once there was a population of cells that when exposed to ethylene oxide exhibited the uniform normal exponential death rate, now we find that the curves for survival, when plotted on a semi-log scale, are no longer linear.

One of the most obvious explanations for these abnormal decay rates is that one is dealing with a nonhomogeneous population. This is quite strikingly suggested in these examples.

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* Phillips, C.R.; unpublished data.

All of the cells are certainly not resistant, since as many as 90 per cent or more appear to be killed at more or less the normal rate. A small percentage of cells, however, exhibit a marked resistance; increasing the time of exposure or the concentration of ethylene oxide does not appear to affect them.

An attempt was made to determine whether the heterogeneous behavior was the result of a population with a continuous series of varied resistances (a population containing cells whose resistances varied from normal to almost completely resistant with all degrees of variation in between) or, alternatively whether a mixture of two types of cells was indicated, with cells either unchanged or changed in some manner to be highly resistant. A mathematical analysis of the data was carried out by Mr. Theodore W. Horner.*

Since it is always possible to support mathematically the continuously varying population hypothesis by choice of a large series of reaction rate constants, the attempt was made to see if the data would support the hypothesis of only two types of cells, normal and resistant, using only two reaction rate constants. The conclusion of this mathematical study was that the two-cell type of population hypothesis cannot be ruled out; thus either hypothesis would fit the observed data.

The only certain conclusions that can be drawn are that when bacterial cells are dehydrated beyond a certain critical point and allowed to rehydrate in an atmosphere of greater relative humidity, they (a) contain a lesser amount of moisture than do cells which are equilibrated to the same relative humidity without prior dehydration; and (b) no longer react uniformly to alkylation or, more exactly, to ethylene oxide sterilization. We cannot tell from these data whether this nonuniform population is a simple mixture of only two populations, normal and resistant, or whether it contains cells of many gradations of resistance. The percentage of the cell population exemplifying resistance is quite low, often only 0.001 or 0.01 per cent of the original population, even when the cells are exposed to a desiccating effect for a considerable time. The highest percentage of resistant cells observed was less than ten per cent. The phenomenon was produced by exposing cells to either a vacuum or a chemically desiccated atmosphere. The effect can be completely reversed or overcome, but to do so in a reasonable time, the cells must be rehydrated by exposure to liquid water, or at least to an atmosphere of essentially 100 per cent RH. Exposures to atmospheres of lower but still quite high relative humidities must be very prolonged if they are to be effective.

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* Associated with Booz-Allen Applied Research, Inc., Bethesda 14, Maryland.

Exactly what is happening is impossible to say. Not enough is known about the position of water molecules or "bound water" in dry but not completely desiccated protein or other complex molecules. One is tempted to speculate that certain cross-linkages are formed through water molecule bridges in these complex molecules. When all or almost all of the water molecules are removed, direct cross-linkages are sometimes established in their place, blocking what might otherwise be alkylation sites. These direct cross-linkages, once established, are then broken only upon complete rehydration.

The phenomenon is real enough that one should avoid such desiccating conditions in standard ethylene oxide sterilization procedures. That this can be done in practice is exemplified by the many successful applications of this technique over the last decade.

A similar and also unexplained phenomenon takes place with heat sterilization. Considerably less time is required to kill microorganisms with moist than with dry heat. This fact has to be taken into consideration in the design of autoclaves if sterilization skips are to be prevented. Knowing that any such phenomenon occurs, even though it is not fully understood, is the first step toward avoiding its effects.

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